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Glyphosate has limited short-term effects on commensal bacterial community composition in the gut environment due to sufficient aromatic amino acid levels[☆]



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ABSTRACT

Recently, concerns have been raised that residues of glyphosate-based herbicides may interfere with the homeostasis of the intestinal bacterial community and thereby affect the health of humans or animals. The biochemical pathway for aromatic amino acid synthesis (Shikimate pathway), which is specifically inhibited by glyphosate, is shared by plants and numerous bacterial species. Several *in vitro* studies have shown that various groups of intestinal bacteria may be differently affected by glyphosate. Here, we present results from an animal exposure trial combining deep 16S rRNA gene sequencing of the bacterial community with liquid chromatography mass spectrometry (LC-MS) based metabolic profiling of aromatic amino acids and their downstream metabolites. We found that glyphosate as well as the commercial formulation Glyfonova[®] 450 PLUS administered at up to fifty times the established European Acceptable Daily Intake (ADI = 0.5 mg/kg body weight) had very limited effects on bacterial community composition in Sprague Dawley rats during a two-week exposure trial. The effect of glyphosate on prototrophic bacterial growth was highly dependent on the availability of aromatic amino acids, suggesting that the observed limited effect on bacterial composition was due to the presence of sufficient amounts of aromatic amino acids in the intestinal environment. A strong correlation was observed between intestinal concentrations of glyphosate and intestinal pH, which may partly be explained by an observed reduction in acetic acid produced by the gut bacteria. We conclude that sufficient intestinal levels of aromatic amino acids provided by the diet alleviates the need for bacterial synthesis of aromatic amino acids and thus prevents an antimicrobial effect of glyphosate *in vivo*. It is however possible that the situation is different in cases of human malnutrition or in production animals.

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1. Introduction

Globally, glyphosate-based herbicides are widely applied in agriculture for the control of weeds and for green burndown (Bøhn et al., 2014). Since the late 1970s, the volume of glyphosate-based herbicides applied world-wide has increased approximately 100-fold, especially after the introduction of genetically modified

plants tolerant to these herbicides (Myers et al., 2016). Application of glyphosate-based herbicides on crops will inevitably result in glyphosate residues and potentially its primary metabolite, aminomethyl phosphonic acid (AMPA), in crops at harvest, which may reach the consumers (Myers et al., 2016). In recent years the biologically significant residue level of glyphosate in food commodities has been much debated. Some studies claim possible negative effects following exposure below the regulatory maximum residue (MRL) and accepted daily intake (ADI) levels (Benedetti et al., 2004; Larsen et al., 2014). In Europe the MRL varies for different crops, and is defined for each product type separately. For barley and oats (grains) the MRL is 30 mg/kg, while the ADI is set to 0.5 mg/kg body weight per day (EFSA, 2015). Humans may be exposed to

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glyphosate residues from consuming fruits, vegetables and other agricultural products as well as from drinking-water. This represents a significant public health concern, fueled by the fact that several studies demonstrate the presence of glyphosate in the urine of the general population (Conrad et al., 2016; Krüger et al., 2014).

When assessing oral toxicity of pesticides including glyphosate, the potential impact on the bacterial community within the gut environment has received very little attention until recently (Jin et al., 2017; Liu et al., 2017; Shehata et al., 2013). The significance of this highly complex ecosystem, collectively termed the gut microbiota, has however attracted immense scientific attention in recent years, due to a growing recognition of its importance in human health (Arrieta and Finlay, 2012; Butel, 2014; Jandhyala et al., 2015). It is also well established that the natural homeostasis of the gut microbiota is sensitive towards external influences, such as antibiotic treatments (Dethlefsen et al., 2008), dietary habits (David et al., 2013) and exposure to xenobiotic compounds (Lai et al., 2016; Nasuti et al., 2016). Modulation of this intricate balance has been associated with several disorders such as metabolic diseases, hepatic, coronary and gastrointestinal diseases (e.g. inflammatory bowel disease) (Sheehan et al., 2015). The link between gut microbiota and host health is partly driven by bacterial breakdown of non-digestible dietary fibers, resulting in the generation of short-chain fatty acids (SCFA) (Russell et al., 2013). The SCFA, including acetic acid, propionic acid and butyric acid, play an important role for human health as they serve as nutrition for enterocytes (Brüssow and Parkinson, 2014), and are involved in both appetite regulation (Byrne et al., 2015) and immune homeostasis (Wu and Wu, 2012). Acetic acid, which is the most predominant of the SCFAs in the gut and is produced by many different bacterial taxa, has been shown to induce anti-inflammatory effects in the colonic epithelium, and to prevent *E. coli* infection (Fukuda et al., 2011).

Already around the time glyphosate was patented as a herbicide (Franz, 1974) it was realized that the active compound could have antibacterial capabilities. Glyphosate specifically inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is a central enzymatic step of the Shikimate pathway of aromatic amino acid biosynthesis in plants as well as some bacteria, algae, fungi and parasites (Clair et al., 2012; McLeod et al., 1998; Priestman et al., 2005). Reports of non-target effects of glyphosate on aquatic and soil-living microorganisms are numerous and show that glyphosate does indeed impact environmental microbial communities, for example by selectively enhancing bacteria belonging to the Gammaproteobacteria and inhibiting *Pseudomonas* spp. and *Acidobacteria* (Newman et al., 2016; Zobiole et al., 2011). Therefore, glyphosate could also potentially modify the composition of the microbial community in the gut, and thereby potentially exert a negative effect on the host organism. According to recent *in vitro* studies, bacterial communities associated with the intestinal environment are indeed susceptible to interference by glyphosate. In one study the minimum inhibitory concentration (MIC) for 23 different bacterial species relevant to the intestinal environment was determined for the glyphosate-containing herbicide Roundup UltraMax®. This study indicated that bacteria commonly recognized as beneficial, including bifidobacteria, enterococci and lactobacilli, were more susceptible to the glyphosate formulation than pathogenic bacteria such as *Clostridium perfringens* and *Salmonella* spp. (Shehata et al., 2013). However, an *in vivo* study of rumen fermentation in sheep fed either corn silage alone or mixed with Roundup Ultra® at a high concentration (0.2% wt/wt) revealed no changes in rumen fermentation parameters (pH, ammonia and volatile fatty acids) following 15 days of treatment (Hüther et al., 2005). In agreement with the latter study, an *in vitro* bovine fermentation model did not reveal any significant effects on

ruminal metabolism or composition of the bacterial communities (Riede et al., 2016). It is important to note that several studies have shown that the effect of glyphosate depends on the way it is formulated. In one *in vitro* study, effects of pure glyphosate and Roundup® (R400) on three food microorganisms *Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* were compared. It was found that Roundup® had an inhibitory effect on microbial growth, but that exposure to glyphosate at the same concentrations had no effect (Clair et al., 2012). Also Braconi et al. (2006) report that pure glyphosate affects cell growth and metabolism in the yeast *Saccharomyces cerevisiae* less than commercial preparations (Braconi et al., 2006).

Overall, the available data from literature is scarce and partly contradictory in terms of the effects of glyphosate on intestinal microbial communities. It remains unclear whether oral exposure to glyphosate residues has the potential to modulate the human gut microbiota at a level of concern for human health. Here, we report on the effects of pure glyphosate and a commercial glyphosate formulation on the intestinal microbial composition, activity and host response during a short-term exposure trial in Sprague Dawley rats. In contrast to previous studies, we address the potentially alleviating effects of endogenous aromatic amino acids in the intestinal environment.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains used in this study are listed in Table 1. All strains were grown in brain heart infusion broth (BHI) (Oxoid) or reinforced clostridial medium (RCM). *Escherichia coli* ATCC 25922 was additionally grown in AB minimal medium (Clark and Maaløe, 1967) containing 2.5 mg thiamine/mL and 0.5% glucose (ABTG) to allow investigation of the effects of aromatic amino acids in the growth media.

2.2. Chemicals

Glyphosate was used in the formulations; Glyphosate (*N*-phosphonomethyl)glycine (Sigma-Aldrich 1071-83-6), Glyphosate salt *N*-(Phosphonomethyl)glycine with monoisopropylamine as counter-ion (Sigma-Aldrich 38641-94-0), Glyfonova® 450 Plus (450 g/L glyphosate acid equivalent) (kind gift from FMC Corporation, previously Cheminova A/S) and Roundup® Garden (120 g/L glyphosate equivalent) (Monsanto). Underlined names are used henceforth.

For the aromatic amino acid analysis, LC-MS grade acetonitrile, methanol, ammonium hydroxide and formic acid were obtained from Merck (Darmstadt, Germany). All aqueous solutions for LC-MS analysis were prepared using ultrapure water obtained from a Milli-Q Gradient A10 system (Millipore, Bedford, MA). Authentic aromatic amino acid compounds (Table S1) including *L*-Tyrosine, *L*-Tryptophan and *L*-Phenylalanine were obtained from Sigma-Aldrich. Aromatic amino acid internal standards (*L*-Phenylalanine (ring-d5, 98%), *L*-Tyrosine (ring-d4, 98%), *L*-Tryptophan (indole-d5, 98%) and indoleacetic acid (2,2-d2, 96%)) of the highest purity grade available were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA).

For the Glyphosate and AMPA analysis: Ammonium acetate, AMPA, ammonia solution 25%, and HPLC-MS grade water were obtained from Sigma-Aldrich (St. Louis, MO). Direct labelled internal standards, glyphosate-2-13C and glyphosate (13C, 99%; 15N, 98%; methylene-D2, 98%), were obtained from Sigma Aldrich and Cambridge Isotope Laboratories Inc. (Andover, MA) respectively.

Table 1

Minimal inhibitory concentration (MIC) towards glyphosate (Glyfonova® 450 PLUS) for selected bacterial strains grown in rich nutrient broth.

Species	Cell type	MIC (mg/mL) BHI	MIC (mg/mL) RCM
<i>Bifidobacterium adolensis</i> DSM 20083	Gram +	10	20
<i>Bifidobacterium bifidum</i> DSM 20456	Gram +	10	20
<i>Bifidobacterium breve</i> DSM 20091	Gram +	10	20
<i>Bifidobacterium longum</i> subsp. <i>infantis</i> DSM 20088	Gram +	10	20
<i>Bifidobacterium animalis</i> DSM 10140	Gram +	10	20
<i>Bifidobacterium animalis</i> lactis BL-04	Gram +	10	20
<i>Clostridium perfringens</i> CCUG 1795	Gram +	10	20
<i>Clostridium leptum</i> DSM 753	Gram +	10	20
<i>Clostridium nexile</i> DSM 1787	Gram +	10	20
<i>Enterococcus faecalis</i> ATCC 29212	Gram +	80	40
<i>Enterococcus faecalis</i> DSM 2570	Gram +	80	40
<i>Lactobacillus johnsonii</i> DSM 10533	Gram +	20	20
<i>Lactobacillus planetarum</i> DSM 20174	Gram +	40	40
<i>Lactobacillus reuteri</i> DSM 20016	Gram +	40	40
<i>Lactobacillus rhamnosus</i> ATCC 53103	Gram +	40	40
<i>Bacteroides uniformis</i> DSM 6597	Gram -	5	10
<i>Bacteroides vulgatus</i> DSM 1447	Gram -	5	20
<i>Bacteroides ovatus</i> DSM 1896	Gram -	10	20
<i>Bacteroides fragilis</i> DSM 2151	Gram -	5	40
<i>Escherichia coli</i> ATCC 25922	Gram -	80	20
<i>Escherichia coli</i> DSM 18039	Gram -	80	20
<i>Akkermansia muciniphila</i> DSM 22959	Gram -	20	20

BHI: Brain Heart Infusion broth, RCM: Reinforced clostridial medium.

2.3. Minimal inhibitory concentration

The broth dilution method was used to determine the lowest concentration of glyphosate that inhibited growth under anaerobic conditions as previously described (Wiegand et al., 2008). Bacteria (Table 1) from overnight (ON) blood agar plates were re-suspended to an $OD_{600} = 0.2$ and further diluted 1000-fold in BHI, RCM or ABTG broth to obtain approximately 10^5 CFU/mL as confirmed in each experiment by plating cell suspensions on agar plates incubated anaerobically ON at 37 °C.

Working solutions of the pesticides were prepared in broth and 100 µL of two-fold dilution series were distributed in 96-well flat bottom microtiter plates (Nunc, ThermoFisher). Subsequently, 100 µL aliquots of the CFU-adjusted bacterial suspensions were transferred to the wells. Positive and negative controls were included in each experiment. Plates were incubated under anaerobic conditions (80% N₂, 10% CO₂ and 10% H₂) at 37 °C and inspected after 24, 48 and 72 h. The MIC-value for each bacterial strain was defined as the lowest concentration of the challenge pesticide formulation giving rise to no visible growth. All experiments were performed in triplicates and repeated twice.

2.4. Bacterial growth

Growth experiments were performed with *E. coli* ATCC 25922 in minimal media (ABTG). The *E. coli* strain was grown anaerobically on blood agar plates and pure cultures were inoculated at $OD_{600} = 0.05$ in ABTG broth in 96 well microtiter plates (Nunc, ThermoFisher). Glyphosate, glyphosate salt, Glyfonova® or Roundup® were added to wells to a final concentrations of 0.04 mg/mL, 0.08 mg/mL and 0.16 mg/mL of the active compound. A mixture of the three aromatic amino acids phenylalanine, tyrosine and tryptophan was added to the ABTG minimal growth media to obtain final concentrations of 0.01 µg/mL, 0.1 µg/mL, 1 µg/mL, 10 µg/mL and 100 µg/mL for all three amino acids. Growth experiments were performed in triplicates and repeated twice.

2.5. Ethical statement

Animal experiments were carried out at the DTU National Food Institute (Denmark) facilities. Ethical approval was given by the

Danish Animal Experiments Inspectorate with authorization number 2012-15-0201-00553 C2. Experiments were overseen by the National Food Institute in-house Animal Welfare Committee for animal care and use.

2.6. Animals, housing and experimental design

4-week old male Sprague Dawley rats ($n = 80$) were purchased from Taconic Biosciences (Lille Skensved, Denmark). Animals had access to *ad libitum* water and feed (Altromin 1324; Altromin Spezialfutter, Lage, Germany) throughout the experiment and were housed under controlled environmental conditions (12-h light/dark cycles, temperature 21.5 ± 0.3 °C, relative humidity $51.3 \pm 3.1\%$, 8–10 air changes per hour). Animal weight was recorded daily during the intervention period.

Upon arrival animals were randomly caged in pairs and acclimatized for 7 days before initiation of the intervention, at which point cages were evenly allocated into four treatment groups based on weight. During the 2-week intervention period animals received water (CTR), glyphosate 2.5 mg/kg/day (GLY5), glyphosate 25 mg/kg/day (GLY50) or Glyfonova® 25 mg/kg/day glyphosate acid equivalent (NOVA) by oral gavage (Fig. 2A). The pH of the glyphosate solutions were adjusted from pH ≈ 2 to pH = 5 using NaOH, as toxicity of glyphosate-based formulations has been found to be influenced by pH (Mann and Bidwell, 1999; Tsui and Chu, 2003) and to minimize any direct effects caused by low pH. Fecal pellets were collected directly from individual rats before the first treatment and immediately frozen at -80 °C. Following the 2-week intervention period, all animals were euthanized by CO₂/O₂ sedation and decapitation, and blood was taken directly for serum preparation. One animal from each cage ($n = 40$) was dissected and inspected for abnormalities. The entire cecum was weighed, and multiple samples of intestinal content from the ileum, cecum and colon were taken and either snap-frozen in liquid N₂ or processed as described below for amino acid profiling.

2.7. Bacterial community composition

Bacterial Community DNA was extracted from 250 mg fecal samples collected on the initial day of intervention and lumen content of ileum, colon and cecum from the day of dissection using

the MoBio PowerLyzer™ PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's recommendations, including bead beating at 30 cycles/s for 10 min (Retsch MM 300 mixer mill). Total DNA concentrations were measured with the Qubit dsDNA HS kit (Life Technologies). The bacterial community composition was determined by sequencing of the hypervariable V3-region of the 16S rRNA gene in the extracted bacterial DNA. Amplification of the V3-region and sequencing using the Ion Torrent PGM platform (Life Technologies) was performed as previously described (Laursen et al., 2016; Tulstrup et al., 2015). Briefly PCR products were obtained by a single fusion-primer PCR strategy using a universal forward primer (PBU 5'-A-adapter-TCAG-barcode-CCTACGGGAGCGCAGCAG-3') with a unique 10–12 bp barcode for each bacterial community (IonXpress barcodes as suggested by the supplier, Life Technologies) and a universal reverse primer (PBR 5'-trP1-adapter-ATTACCGGGCTGCTGG-3'). The PCR reactions contained 5 ng community DNA as template, 0.2 µL Phusion® High-Fidelity DNA Polymerase (New England Biolabs Inc.), 4 µL HF-buffer, 0.4 µL dNTPs (10 mM of each), 1 µM forward primer and 1 µM reverse primer in a total volume of 20 µL. The PCR amplification program included 30 s at 98 °C, 24 cycles of 15 s at 98 °C and 30 s at 72 °C, final elongation for 5 min at 72 °C and then cooling to 4 °C.

The PCR products were subsequently purified with HighPrep PCR magnetic beads (Magbio) with a 96-well magnet stand (Magbio) according to manufactures recommendations. The DNA concentrations were measured using Qubit dsDNA HS assay (Invitrogen) and samples were pooled in equimolar concentrations to obtain two separate libraries containing samples originating from either ileum and cecum or colon and feces (before intervention). Prior to DNA extraction and library preparation, samples were randomized between treatment groups.

Sequencing was performed by the DTU in-house facility (DTU Multi-Assay Core (DMAC), Technical University of Denmark on a 318-chip for Ion Torrent sequencing using the Ion OneTouch™ 200 Template Kit v2 DL generating a median length of 180 bp. Sequencing data were imported into and further processed in CLC Genomic Workbench (version 8.5, Qiagen, Aarhus, Denmark) in order to de-multiplex, trim and remove barcodes and PCR primers (retaining reads only if both forward and reverse primers were present with 100% homology) and further discard any read below 110 bp or above 180 bp. Operational Taxonomic Units (OTUs) were picked *denovo* using UPARSE (Edgar, 2013) with a maximum expected error rate (maxee) set at 1.5, and an OTU table was generated. Taxonomy was assigned using the Ribosomal Database Project multiclassifier version 2.10.1 and the RDP database (Wang et al., 2007) with confidence threshold set to 0.5 as recommended for sequences shorter than 250bp (Claesson et al., 2009). Further downstream processing was performed in QIIME (Caporaso et al., 2010). A phylogenetic tree was generated (make_phylogeny.py) and rooted to an Archaea species following alignment of all OTUs. The OTU table was filtered to include only OTUs assigned as bacteria, excluding the Cyanobacteria/Chloroplast group and OTUs with average relative abundance below 0.005% of the total community (Bokulich et al., 2013), resulting in a total of 547 OTUs. The QIIME script core_diversity_analysis.py was used to generate alpha diversity indices and relative abundance at different taxonomical levels with a rarefied depth of 13,000 reads. Sequencing data are deposited at NCBI under BioProject accession number PRJNA412959.

2.8. Short chain fatty acid (SCFA) content and pH of intestinal samples

Cecum content from each group of animals was analysed for acetic acid, propionic acid, butyric acid and valeric acid by GC-MS

(MS-Omics, Denmark). Briefly, 2 µL milliQ water/mg feces were added to each tube, homogenized using ultra sonication and acidified with HCl before centrifugation. Supernatants were transferred to GC-vials and internal standards were added. Raw GC-MS data were processed with software based on the PARAFAC2 model and quantified values are calculated assuming that 1 mg feces corresponds to a volume of 1 µL (Zhao et al., 2006).

Fecal samples collected on the last day of the intervention period were diluted 1:1 in sterile water, vortexed and centrifuged for 10 min at 13,000 rpm. Supernatants were transferred to clean tubes and the pH was determined (Orion™ 3-star benchtop pH meter, ThermoFisher Scientific) in a randomized order after calibration.

2.9. Glyphosate and Aminomethylphosphonic acid (AMPA) in intestinal samples

Glyphosate and AMPA calibration standards were prepared in HPLC grade water at 1 mg/mL and dilutions hereof. Approximately 0.2 g of intestinal sample was transferred into a plastic vial, weighed and diluted with 10 mL of HPLC-grade water. The mixture was homogenized by vortexing, vigorously hand-mixing and sonication for 15 min. Subsequently vials were centrifuged for 20 min at 6000 rpm and 1 mL of supernatant was transferred to an HPLC vial. A total of 50 µL of stable isotope labelled internal standard solutions (10 µg/mL of each) were added to each sample and to calibration standards of glyphosate and AMPA. Intestinal samples were analysed in separated batches for ileum, cecum and colon samples. In each batch, the samples were analysed by increasing order of concentration to avoid possible carry-over of the analytes.

For the separation of glyphosate and AMPA, HILIC chromatography was performed using a Luna NH2 column (3 µm, 50 × 2 mm). The aqueous mobile phase consisted of water containing 10 mM of ammonium acetate adjusted to pH 10 with 25% ammonia solution. The organic mobile phase was acetonitrile. A volume of 10 µL of sample was injected into the HPLC-MS/MS, equipped with a dual low-pressure mixing ternary-gradient system, Dionex UltiMate 3000 (Thermo Scientific). The mass spectrometer was an API 4000 (ABSciex, Framingham, MA, USA) and was operated with an ESI source in positive mode which was set at 400 °C with a capillary voltage of 5500 V.

Glyphosate and AMPA were detected and quantified in MRM mode (multi reaction monitoring). Labelled internal standards were used for quantification. The obtained data was treated with Analyst 1.6 Software. The calibration curves were established by plotting the peak area ratios between analytes and internal standards against the concentrations of the calibration standards. The calibration curves were fitted to a linear regression with weighting factor of 1/x. The correlation coefficient was above 0.99 for all regressions.

2.10. Aromatic amino acids and metabolite profiling

Stock solutions (1 mg/mL) of 23 aromatic amino acids and derivatives as well as four internal standards (IS) were individually prepared from their authentic compounds in water, methanol or 50% methanol as listed (Table S1). An IS solution containing all four stable isotope standards (4 µg/mL each) was prepared. The 23 aromatic amino acids and derivatives were mixed (standard mix) and further diluted with water to achieve final concentrations of 0.1 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL and 4 µg/mL. For preparation of the calibration curves, 100 µL of the IS solution was placed into Eppendorf tubes and dried with nitrogen. Subsequently, 100 µL of the standard mix (for each standard mix concentration) were added to each tube.

During dissection of animals ($n = 40$), intestinal content of ileum, cecum and colon (200–500 mg) was immediately diluted 1:2 with sterile milliQ water, vortexed for 10 s and centrifuged twice at 16,000 g, 4 °C for 5 and 10 min respectively with transfer of supernatant between steps. Finally, an aliquot of 300 μ L was stored at –20 °C until analysis. For analysis, samples were thawed at 4 °C, centrifuged at 16,000 g, 4 °C for 5 min, and the supernatants were diluted in a total volume of 80 μ L water corresponding to a 1:100 dilution of ileum content and a 1:5 dilution of cecum or colon content. Quality control (QC) samples for ileum, cecum and colon were prepared by pooling 10 μ L of each sample from the three respective compartments. To each sample, 20 μ L IS (4 μ g/mL) and 240 μ L of acetonitrile were added. The tubes were vortexed for 10 s and left at –20 °C for 10 min to precipitate the proteins, after which the tubes were centrifuged at 16,000 g, 4 °C for 10 min and each supernatant (320 μ L) was transferred to a new tube, which was dried with nitrogen gas. Subsequently, the residues were reconstituted in 80 μ L water, vortexed for 10 s, centrifuged at 16,000 g, 4 °C for 5 min, and transferred to LC vials, which were stored at –20 °C until analysis.

Intestinal samples from each compartment were analysed separately in random order with a QC sample of the given compartment injected before and after every ten samples throughout the analysis. The five standard mix solutions were also analysed once for every 10 samples. For each sample, a volume of 2 μ L was injected into an ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) system consisting of Dionex Ultimate 3000 RS liquid chromatograph (Thermo Scientific, CA, USA) coupled to a Bruker maXis time of flight mass spectrometer equipped with an electrospray interphase (Bruker Daltonics, Bremen, Germany) operating in positive mode. The analytes were separated on a Poroshell 120 SB-C18 column with a dimension of 2.1 \times 100 mm and 2.7 μ m particle size (Agilent Technologies, CA, USA) based on the previously suggested settings (Want et al., 2010). Briefly, the column was held at 40 °C and the sampler at 4 °C. The UPLC mobile phases consisted of 0.1% formic acid in water (solution A) and 0.1% of formic acid in acetonitrile (solution B). While maintaining a constant flow rate of 0.4 mL/min, the analytes were eluted using 1% solution B for 1 min followed by a linear gradient up to 15% at 3 min, then a linear gradient up to 50% B at 6 min, and finally a linear gradient up to 95% solution B at 9 min, which was held constant until 10 min, followed by a return of the solvent composition to initial conditions at 10.1 min and re-equilibration until 13 min. Mass spectrometry data were collected in full scan mode at 2 Hz with a scan range of 50–1000 mass/charge (m/z).

The following electrospray interphase settings were used: Nebulizer pressure 2 bar, drying gas 10 L/min, 200 °C, capillary voltage 4500 V. For MS/MS analyses, a ramp collision energy ranging from 10 to 30 eV was applied on a scheduled precursor list. To improve the measurement accuracy, external and internal calibrations were done using sodium formate clusters (Sigma-Aldrich, Schnelldorf, Germany), and in addition a lock-mass calibration was applied (hexakis (1H,1H, 2H-perfluoroethoxy) phosphazene, Apollo Scientific, Manchester, UK).

Aromatic amino acids and derivatives were detected by selected ions and quantified by isotopic IS with similar molecular structures as listed in Table S1. Data were processed using QuantAnalysis version 2.2 (Bruker Daltonics, Bremen, Germany) and bracket calibration curves for every 10 intestinal samples were obtained for each compound. The calibration curves were established by plotting the peak area ratios of all of the analytes with respect to the IS against the concentrations of the calibration standards. The calibration curves were fitted to quadratic regression with weighting factor of the reciprocal of the squared concentration ($1/x^2$). The

correlation coefficient was above 0.98 for all regressions. In ileum samples, only phenylalanine, tryptophan and tyrosine were quantified.

2.11. Analysis of host responses

The acute phase protein, haptoglobin, was measured in blood serum in random order using “PHASE”™ Haptoglobin Assay (Tri-delta, Kildare, Ireland) according to the manufacturer's recommendations. The microtiter plates were read at 600 nm in a multi label plate reader (VICTOR™ $\times 3$, PerkinElmer, MA.). Samples were measured in duplicates and concentrations were calculated from a standard curve.

The level of IL-6 in blood serum was quantified in random order using a specific sandwich ELISA kit (rat) (Cusabio Biotech) and a microplate reader (BioTek) at 450 nm, with a range of detection between 0.312 pg/mL and 20 pg/mL according to the manufacturer's recommendations.

2.12. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 7.01 (GraphPad Software, Inc. CA) or R (version 3.1.0) (R Core Team, 2013). Differences between treatment groups were assessed by use of un-paired *t*-tests or non-parametric Mann-Whitney tests, if variances were found to be different. Differences between intestinal compartments were assessed by use of paired *t*-tests or Wilcoxon tests as appropriate. Correlation analysis was performed using Spearman's rank test. For all statistical analysis *p*-values less than 0.05 were considered significant. For multiple comparisons of bacterial groups at the genus level permutation based *t*-tests were applied. For assessing differences between aromatic amino acid metabolites in the treatment groups, Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison post-test was used. In both of these cases, correction for multiple testing was applied using the false discovery rate method with a threshold of $q < 0.05$ (Pike, 2011). The metabolite data were imported into LatentX (version 2.11) (Latent5) for principle component analysis (PCA) to assess the quality of the data. QC samples clustered tightly in PCA score plots indicating a stable system.

3. Results

3.1. Minimal inhibitory concentrations

Minimal inhibitory concentrations (MIC) were determined for a selected group of bacteria representative of the gut microbiota in humans. Two different nutrient rich media, namely BHI and RCM, were chosen that both supported growth of all the included strains under identical growth conditions. The commercial formulation Glyfonova® was used as test compound because of its high solubility compared to glyphosate. Overall, all bacterial species tested showed very high MIC values in both growth media ranging between 5 mg/mL to 80 mg/mL, although some variability between bacteria and media was found (Table 1).

3.2. Importance of aromatic amino acids

Because glyphosate inhibits aromatic amino acid synthesis (Geiger and Fuchs, 2002), we investigated the importance of bioavailable aromatic amino acids in the growth media by determining the MIC value for *E. coli* ATCC 25922 in ABTG minimal growth medium with or without supplementation of a mixture of phenylalanine, tyrosine and tryptophan. With no added aromatic amino acids a MIC of 0.08 mg/mL towards Glyfonova® was

determined. Supplementation of the growth medium with either 50 µg/mL or 500 µg/mL of tryptophan, phenylalanine and tyrosine increased the MIC to 10 mg/mL and 20 mg/mL, respectively (data not shown).

To further investigate the importance of aromatic amino acids in the growth medium, 24-h growth experiments were set up with *E. coli* ATCC 25922 grown anaerobically in minimal medium containing 0.08 mg/mL Glyfonova® (equal to the MIC in ABTG medium without added amino acids) supplemented with 0 µg/mL, 0.01 µg/mL, 0.1 µg/mL, 1 µg/mL, 10 µg/mL or 100 µg/mL aromatic amino acid mix. Bacterial growth was determined as OD₆₀₀ every hour (Fig. 1A). The results revealed a clear dose-dependent alleviation of the inhibitory effect of Glyfonova® with aromatic amino acid supplementation, however even at the highest concentration a noticeable lag-phase before growth initiation was observed compared to the control group (no Glyfonova®) although the maximum growth rates (slope) were comparable. A concentration between 0.1 µg/mL and 1 µg/mL aromatic amino acid mix was required to allow growth of *E. coli* ATCC 25922 (Fig. 1A).

3.3. Impact of different glyphosate formulations on growth inhibition

The 24-h growth experiments of *E. coli* ATCC 25922 in minimal ABTG medium with different glyphosate formulations in the absence of aromatic amino acids showed, that Glyfonova® had the strongest effect on growth, with an apparent slight lag-phase observed in the presence of 0.04 mg/mL (Fig. 1B) and a complete inhibition of growth during 24-h at the established MIC of 0.08 µg/mL (Fig. 1C). At a concentration of 0.08 mg/mL all other

formulations of glyphosate resulted in growth of the *E. coli* strain during the 24-h period after an initial lag-phase compared to the control (Fig. 1C). The active compound in its pure form (Glyphosate) had a significantly lower impact on growth than the three other formulations as determined by the area under the curve (0.08 µg/mL glyphosate) during 24-h of growth ($p < 0.01$). All the tested formulations inhibited *E. coli* ATCC 25922 completely at a concentration of 0.16 mg/mL.

3.4. Effect of glyphosate on short-term weight gain in rats

After two weeks of exposure to Glyphosate or Glyfonova® (Fig. 2A), no significant differences in rat body weight gain during the intervention period, or in cecum weight at termination, were observed between any of the treatment groups as compared to the control group (Fig. 2B and C).

3.5. Levels of glyphosate and AMPA in ileum, cecum and colon

A clear dose-dependent relation between the concentration of orally administered glyphosate and detected levels of glyphosate in all three intestinal compartments was observed, with the colon compartment containing the highest concentration of glyphosate ($p < 0.0001$) compared to both cecum and ileum (Fig. 2D). The detection of both glyphosate and AMPA in intestinal samples from animals in the control group was explained by low residues being present in the standard animal feed (Glyphosate: 1.0 ± 0.03 µg/g and AMPA: 0.72 ± 0.12 µg/g). As glyphosate was orally administered for the last time one day prior to dissection and collection of samples, the actual concentrations of glyphosate/AMPA in the

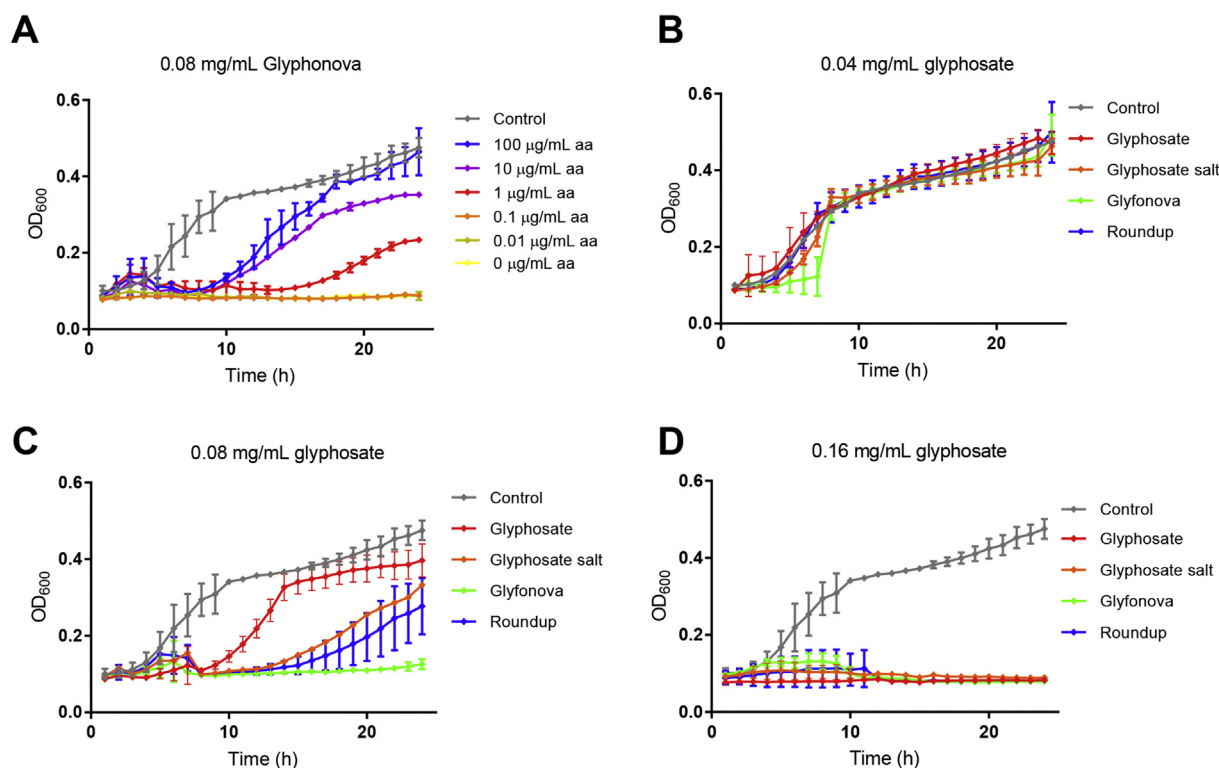


Fig. 1. (A) *Escherichia coli* ATCC 25922 grown in ABTG minimal medium containing 0.08 mg/mL Glyfonova®450 Plus supplemented with a mix of three aromatic amino acids (tyrosine, phenylalanine and tryptophan) at different concentrations. The control contains neither Glyfonova® nor amino acids. (B–D) Effect of glyphosate, glyphosate-isopropylamine salt, Glyfonova®450 Plus and Roundup® on *E. coli* ATCC 25922 growth in ABTG minimal medium supplemented with (B) 0.04 mg/mL, (C) 0.08 mg/mL and (D) 0.16 mg/mL active compound. Data are presented as means with error bars showing SD.

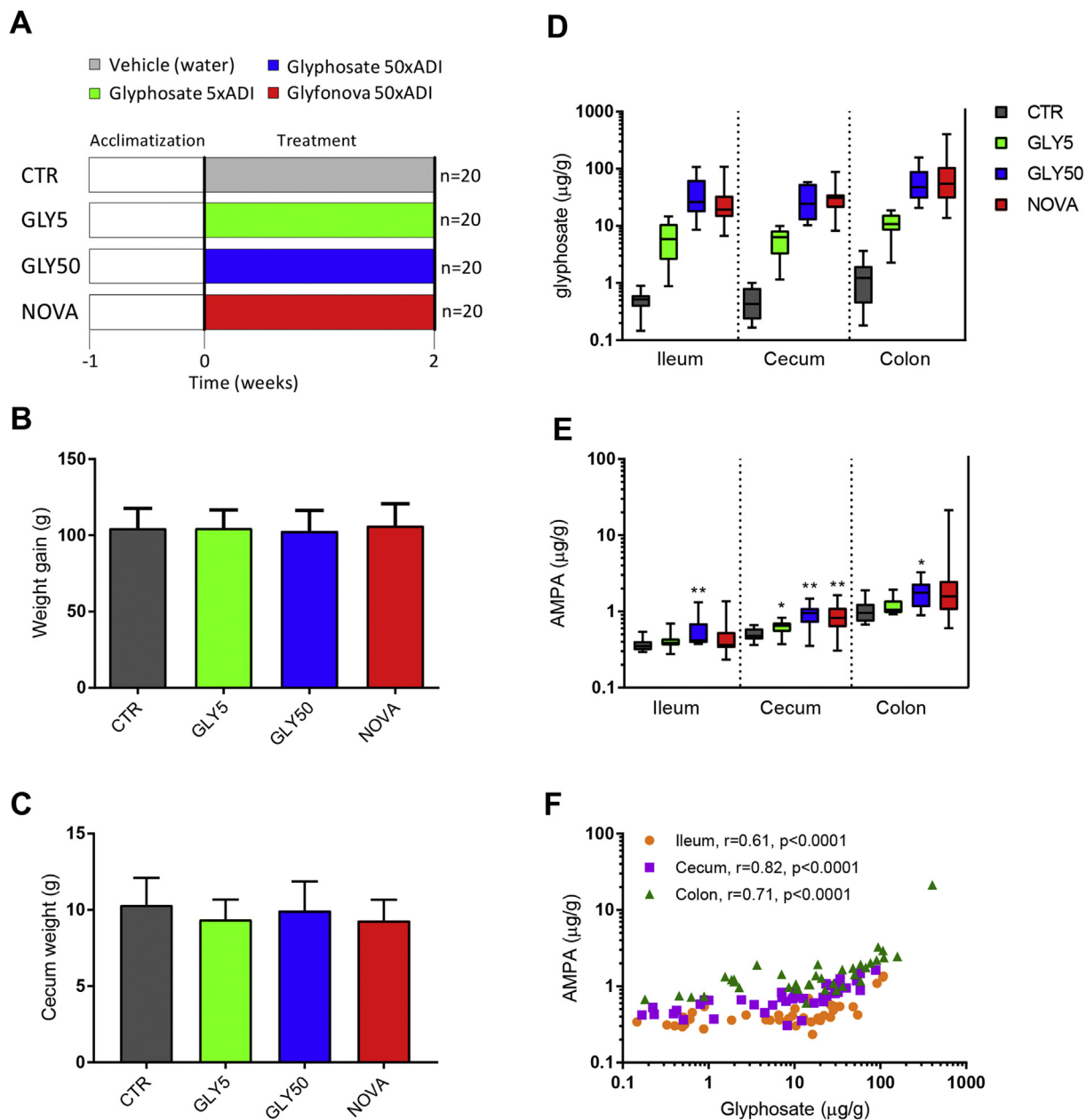


Fig. 2. (A) Study design. Animals were caged in pairs and acclimatized for one week before receiving glyphosate, either in pure form or as a commercial formulation, by oral gavage daily for a period of two weeks. Fecal samples were taken before the first treatment and intestinal and blood samples were collected at termination. (B) Weight gain of rats during the treatment period. (C) Weight of cecum with content at termination. (D) Glyphosate and (E) AMPA concentrations (µg/g) in ileum, cecum and colon. (F) Spearman correlation analysis between glyphosate and AMPA concentrations in ileum, cecum and colon. For each compartment the Spearman r and p -value is shown. Data in (B–C) are presented as means with error bars showing SD. Data in (D–E) are presented as box-plots with whiskers showing the total range.

intestinal tract during the intervention period may have been higher than measured in intestinal samples. The primary degradation product of glyphosate, AMPA, was detected at levels above the background levels (CTR group) in all three compartments, but significant differences for the lowest treatment group (GLY5) were only detected in the cecum (Fig. 2E). Overall the concentration of AMPA was highest in the colon, followed by cecum and then ileum ($p < 0.0001$ for all pair-wise comparisons). Significant correlations were found between the level of glyphosate and the concentration of AMPA in the same animal in all three compartments ($p < 0.0001$), and the AMPA to glyphosate ratio appeared to increase through the intestinal tract from the ileum through cecum to the colon (Fig. 2F).

3.6. Fecal pH and levels of short chain fatty acids in the cecum

The pH in feces was measured in samples taken on the last day of the intervention period and showed a significantly higher pH in all three treatment groups compared to the CTR group (Fig. 3A). Concentrations of the SCFAs acetic acid, propionic acid, butyric acid and valeric acid were determined in cecum (Fig. 3B). The level of acetic acid was significantly lower ($p = 0.01$) in animals in the NOVA group compared to CTR, and the same tendency was also noted for the GLY50 group ($p = 0.06$). A strong positive correlation was found between colonic levels of glyphosate and fecal pH (Fig. 3C), whereas a weaker positive correlation was found between colonic levels of AMPA and fecal pH (data not shown). Conversely, a

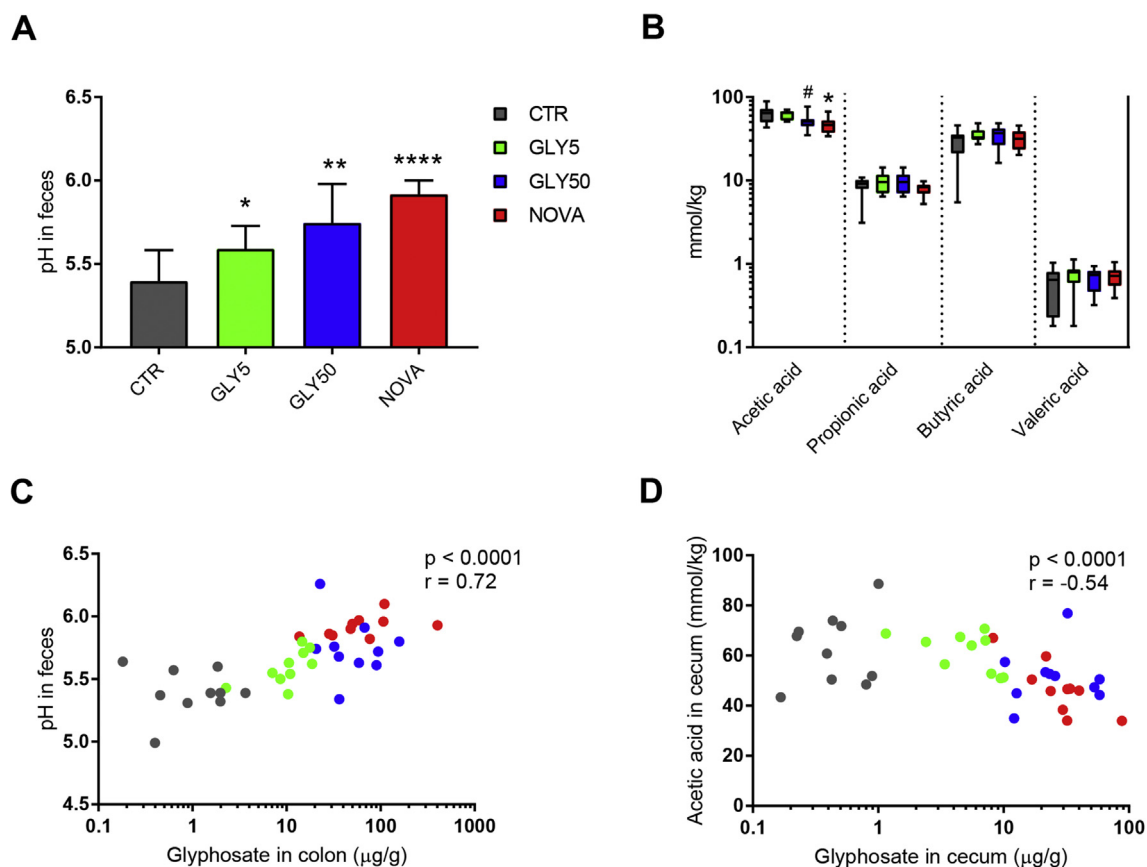


Fig. 3. (A) Fecal pH after the intervention period presented as means with error bars showing SD. (B) Concentration in cecum of the SCFAs; acetic acid, propionic acid, butyric acid and valeric acid presented as box-plots with whiskers showing the total range. Spearman correlation analysis between (C) colonic glyphosate concentration and fecal pH and (D) glyphosate and acetic acid concentration in the cecum. In panels (A–B) * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; # $p < 0.1$. For correlations (C–D) the p -value and Spearman r is shown.

negative correlation between cecal glyphosate and cecal acetic acid was found ($r = -0.54$, $P < 0.0001$) (Fig. 3D), which was not found for the other SCFAs. Additionally, fecal pH and the concentration of acetic acid in cecum correlated negatively ($r = -0.58$, $p < 0.0001$).

3.7. Changes in bacterial diversity and community composition following glyphosate exposure

In all groups of animals, both the number of observed species (OTUs) and the Shannon diversity index were lower in the ileum as compared to the cecum and colon (Fig. 4A and B). A significantly higher number of OTUs was found in the cecum and colon of the NOVA group as compared to the control group (Fig. 4A). In the cecum, also a higher Shannon diversity index was found in the NOVA group (Fig. 4B). A significant difference in numbers of observed species between the GLY50 and NOVA groups, both treated with the same concentration of active compound, was also noted in both the cecum and the colon (Fig. 4A) and also no effect of the active compound (GLY5 and GLY50 groups) on alpha diversity was observed based on *in situ* measurements of glyphosate (Fig. 4C). Overall, the bacterial communities in all compartments were dominated by classes within the phyla Firmicutes, Bacteroidetes and Actinobacteria. Bacterial composition at the class level did not reveal differences between the treatment groups and the control group in any of the three compartments tested (ileum, cecum and colon), nor were differences observed in the fecal bacterial communities before the intervention (Fig. 4D). Analysis of differences between the treatment groups and the control group at the genus level indicated only two significantly differently

abundant genera after correcting for multiple comparisons, namely *Clostridium sensu stricto* and *Ruminococcus*, of which the former was found to be different between the CTR and NOVA groups before the intervention (Fig. 4E). Large differences in community composition between the different compartments were seen, irrespective of treatment group (Fig. 4D and E).

3.8. Aromatic amino acids and metabolic profiling

Since glyphosate is known to inhibit the Shikimate pathway (Geiger and Fuchs, 2002), the colonic levels of the three aromatic amino acids (tyrosine, phenylalanine and tryptophan) as well as their derivatives were measured (Fig. 5A). The highest levels of all three aromatic amino acids were found in the ileum irrespective of treatment group (Fig. 5B–D). Both tyrosine and phenylalanine concentrations were significantly lower in the cecum than in the colon, and a tendency for this was also seen for tryptophan (Fig. 5B–D). A single significant difference in levels of aromatic amino acids was found for tyrosine in the ileum of the NOVA group (but not the GLY50 group) as compared to the CTR group. No differences between treatment groups and the control group were found for any of the downstream metabolites determined in cecum and colon after correcting for multiple testing (Fig. 5E).

3.9. Haptoglobin and IL-6 levels in blood serum

No differences were found in levels of serum IL-6 in any of the treatment groups as compared to the CTR group (Fig. 6A). Serum levels of the acute phase protein haptoglobin were significantly

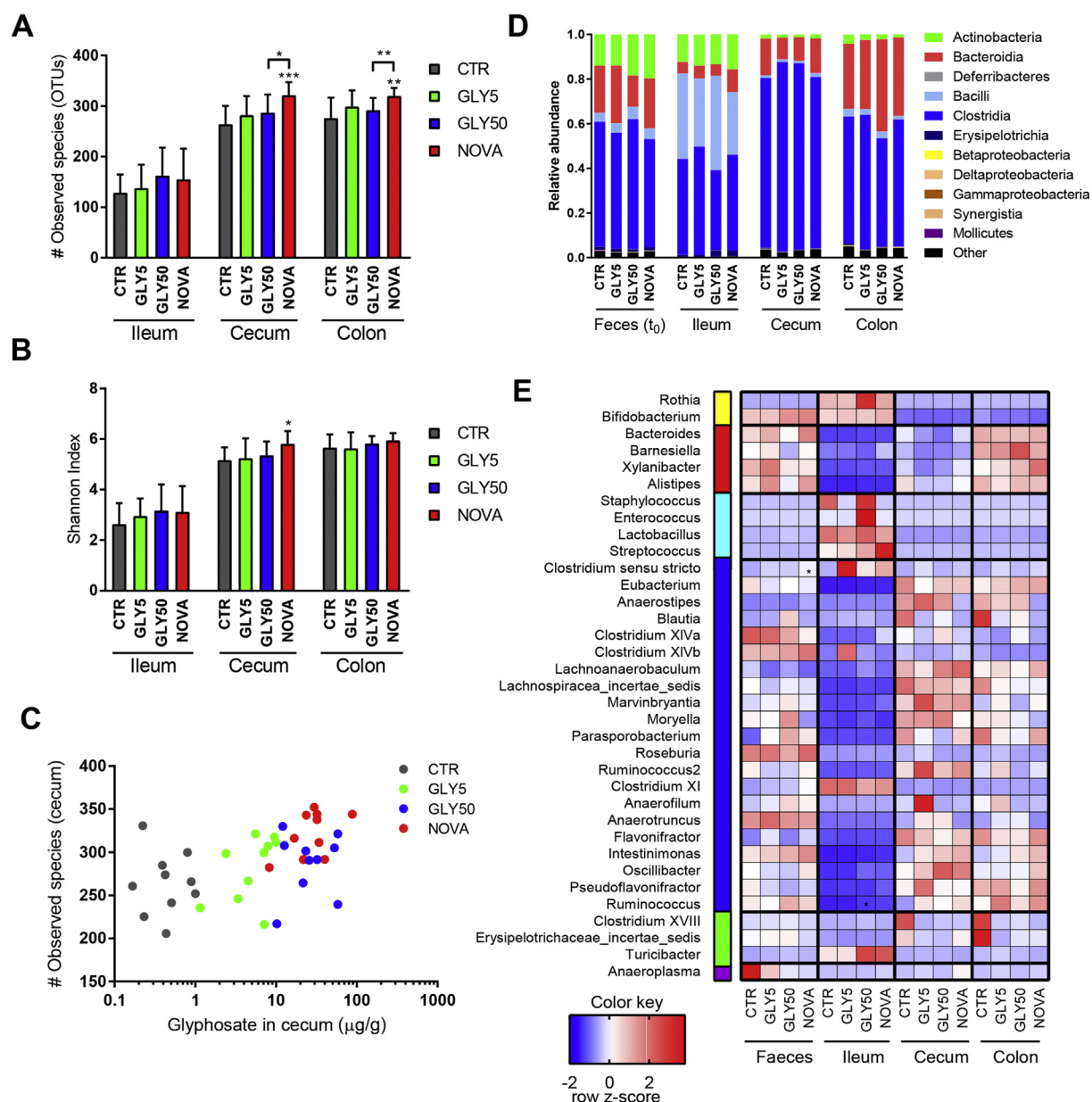


Fig. 4. (A) Number of observed species (OTUs) and (B) Shannon diversity index for each group and compartment are shown as mean with error bars showing SD. In panels (A–B) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (C) Correlation analysis between glyphosate concentration and number of observed species in cecum. (D) The mean bacterial composition at the class level in feces (t₀: Before intervention), ileum, cecum and colon. Bacterial classes with prevalence less than 25% across all samples and bacterial groups not classified to at least the class level were aggregated into the category: Other. (E) Heat-map showing average relative abundance of genera for each group of animals in feces (before intervention), ileum, cecum and colon, respectively. Colours indicate row z-scores. The bacterial genera shown represent above 1% of the community in any single sample and are present in at least 25% of all samples. Significant differences between the control group and the treated groups are shown as * $q < 0.05$ (FDR corrected permutation based t -test). The left-hand colour bar shows the classification of bacterial genera (Yellow: Actinobacteria, Red: Bacteroidetes, Cyan: Bacilli (Firmicutes), Blue: Clostridia (Firmicutes), green: Erysipelotrichia (Firmicutes) and purple: Tenericutes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

higher in the NOVA group as compared to the CTR group (Fig. 6B).

4. Discussion

The *in vitro* susceptibility of selected bacterial strains associated with the intestinal environment to glyphosate (Table 1) revealed higher MIC values than previously reported by others (Clair et al., 2012; Shehata et al., 2013). However, to some extent our results support the previous finding that different bacterial groups have different susceptibility towards glyphosate. Specifically, we found that species belonging to *Bacteriodes* and *Bifidobacterium* were

more sensitive towards a commercial glyphosate formulation than *E. coli*, although the differences observed were much smaller than previously reported (Shehata et al., 2013). The discrepancies between studies may in part be due to the use of various growth media and growth conditions as well as different formulations of glyphosate. In this study we used a formulation without Polyethoxylated tallow amine (POEA), a compound which may potentially have increased the effect on bacteria in the study by Shehata et al. (2013). Additionally, the very high MIC values observed in the present study compared to previously reported may reflect the use of growth media containing high levels of free aromatic amino

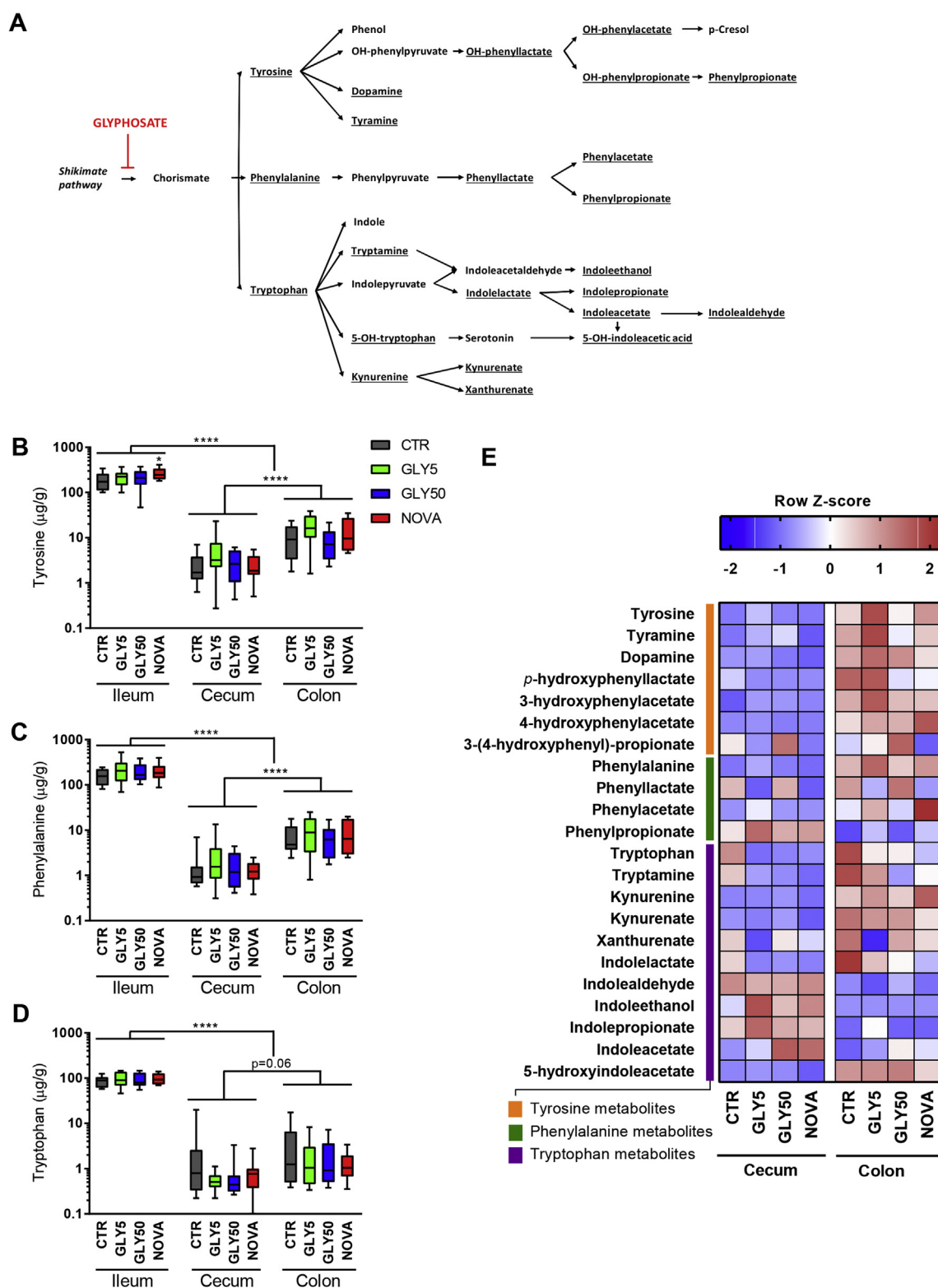


Fig. 5. (A) Outline of how glyphosate potentially affects the catabolism of aromatic amino acids in the intestine. Underlined metabolites are those targeted in the present study. Concentrations of (B) tyrosine, (C) phenylalanine and (D) tryptophan in ileum, cecum and colon in treatment groups CTR, GLY5, GLY50 and NOVA are shown. Data are presented as box-plots with whiskers showing full range. In panels (B–D) * $p < 0.05$; **** $p < 0.0001$. (E) Heatmap showing mean concentration (z-score) of aromatic amino acids and derivatives in cecum and colon, collectively. No differences between the CTR group and the treatment groups were found for any of the metabolites after correcting for multiple testing.

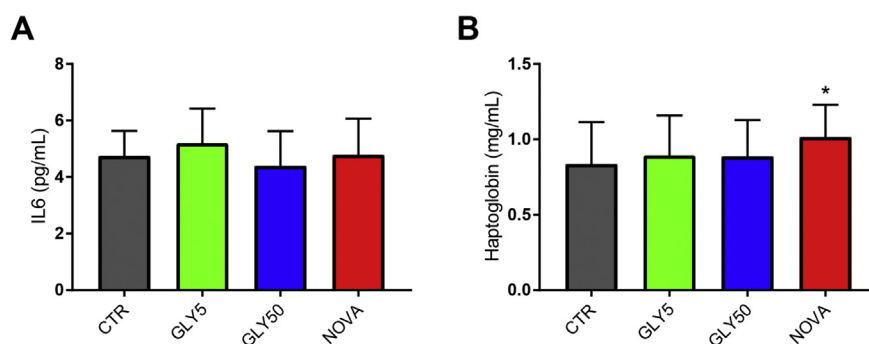


Fig. 6. Concentration of (A) IL-6 and (B) haptoglobin in blood serum from rats in treatment groups CTR, GLY5, GLY50 and NOVA. Data are presented as means with error bars showing SD. * $p < 0.05$.

acids, which has been shown to alleviate the susceptibility of bacteria towards glyphosate (Gresshoff, 1979; Haderlie et al., 1977) due to redundancy of the Shikimate pathway. In support of this, we found that *E. coli* ATCC 25922 had a significantly lower MIC value (0.08 mg/mL) in minimal medium without added amino acids than in rich growth medium. This was further substantiated by 24-h growth experiments, which showed that adding the three aromatic amino acids tyrosine, tryptophan and phenylalanine reduced the inhibitory effect of glyphosate in a dose dependent manner, with no growth detected at or below 0.1 μ g/mL aromatic amino acids in the growth medium (Fig. 1A).

The active compound glyphosate in commercial products for application in agriculture is mostly formulated as isopropylamine (IPA) salt, although other cations such as diammonium, potassium, trimesium, and sesquisodium are also used (Lee et al., 2009). Additionally, additives that increase water solubility and uptake in the plant may be used. The *in vitro* results from the present study support the notion that different formulations of glyphosate may affect bacteria differently. We found that pure glyphosate had a significantly lower impact on growth of *E. coli* than both the glyphosate IPA salt, the commercial formulations Glyfonova® and Roundup® during exposure to the same active compound concentration of 0.08 mg/mL (Fig. 1C). However at half this concentration (0.04 mg/mL), largely no impact on growth was observed for any of the formulations (Fig. 1B) and at double the concentration (0.16 mg/mL) all formulations effectively inhibited growth of *E. coli* during the 24-h growth experiment (Fig. 1D).

To investigate the *in vivo* effects of glyphosate on intestinal bacteria, we applied an animal model with daily oral administration of glyphosate during a two-week period. The relatively short exposure period reflects the expected fast response of the microbiota to an antimicrobial compound (Tulstrup et al., 2015). The exposure of animals to 5x and 50x ADI respectively was chosen to evaluate current threshold limit values for the herbicide. Overall, we found no effects of the active compound on alpha diversity and bacterial community composition when comparing the GLY5 and GLY50 groups to the control group in any of the intestinal compartments (Fig. 4). The lack of modulation of the gut bacterial community *in vivo* is likely caused by the presence of sufficient levels of intestinal aromatic amino acids to alleviate the effect of blocking the Shikimate pathway. This appears to be different from the situation in soil environments, where the bioavailability of aromatic amino acids is probably low and where glyphosate has been demonstrated to cause a shift in bacterial community composition (Newman et al., 2016). Indeed, evidence from genomic investigations supports this view, as most of the free-living bacteria in the soil environment appear to contain a complete and functioning Shikimate pathway, whereas for host-associated bacteria in

the gut environment, more than one-quarter have incomplete pathways, suggesting that they have access to sufficient amounts of aromatic amino acids and thus no need to spend energy on prototrophic synthesis (Zucko et al., 2010).

To confirm the presence of sufficient aromatic amino acids levels in the intestine, we determined the concentrations of tyrosine, phenylalanine and tryptophan in ileum, cecum and colon. We found the highest levels of all three amino acids in the ileum, while lower levels were detected in cecum and colon (Fig. 5B–D), which is consistent with the expected uptake of amino acids in the small intestine (Stevens et al., 1984). No differences between Glyphosate treated animals (GLY5 and GLY50) and control animals were found in any of the intestinal compartments, however a slightly higher level of tyrosine in the ileum was found in the NOVA group. Furthermore, no differences in any of the downstream glyphosate metabolites were observed between groups (Fig. 5E). Thus, it appears (i) that glyphosate treatment in the doses given does not have notable effects on the aromatic amino acid levels in the intestinal environment, and (ii) that the concentrations in general are at a level where no effect would be expected, at least if *E. coli* is representative for other intestinal bacteria (Fig. 1A). We do however find that the aromatic amino acid concentration in the cecum and colon of animals within the same treatment group ranges more than 10-fold (Fig. 5B–D), and may in some animals be sufficiently low to allow inhibition of bacterial growth by glyphosate.

Even though no differences between specific genera were detected, we found that the α -diversity of the bacterial community increased slightly but significantly after treatment with Glyfonova®, both in terms of OTU richness and of the Shannon diversity index (Fig. 4A and B). Such an effect is not consistent with glyphosate functioning as an antimicrobial agent in the gut, as this would normally result in a decrease in α -diversity (Tulstrup et al., 2015) and would also be expected to have been similar in the GLY50 group with the same concentration of the active compound. Thus the Glyfonova® formulation may contain other bacterial nutrient sources to enrich the observed community. Contamination of Glyfonova® with bacterial DNA was also considered a possibility, although the product was confirmed to be free of any bacterial contaminants.

Even though no major changes were observed in bacterial community composition after treatment with 50x ADI of glyphosate or Glyfonova®, we observed significant differences in fecal pH and in levels of acetate in the cecum between treatment groups (Fig. 3A and B) and a strong correlation between colonic glyphosate and fecal pH (Fig. 3C). The mean acetic acid concentration was significantly lower in the NOVA group as compared to the control group, and a tendency was also noted for the GLY50 group. This may at least in part explain the increase in pH observed during

glyphosate treatment, and is consistent with the observed negative correlation between glyphosate and acetic acid in the cecum (Fig. 3D). These observations are interesting because the production of SCFAs by bacteria plays an important role in bacterial-host interaction, influencing energy regulation, appetite and host immune regulation (Comalada et al., 2006; Fukuda et al., 2011). The SCFAs may also affect the bacterial community in the colon by affecting colonic water adsorption and decreasing fecal pH (den Besten et al., 2013). We found no differences between treatment groups in the fecal consistency (data not shown), which is related to transit time and community composition (Roager et al., 2016). Interestingly, a recent investigation of the effects of the pyrethroid pesticide permethrin on the fecal microbiota in rats also revealed changes in levels of SCFAs including acetic acid, and furthermore shifts in microbiota composition including reduced Bacteroidetes and increased Enterobacteriaceae and Lactobacillus (Nasuti et al., 2016). The authors determined the MIC of permethrin towards bacteria and yeast species relevant to the gut environment and found values ranging from 0.4 to 3.2 µg/mL, which is much lower than the values determined for glyphosate in the present study.

We did not observe any physiological abnormalities in organs from the rats dosed with glyphosate. However, we found slightly increased serum levels of the acute phase protein haptoglobin in rats treated with Glyfonova®, however not with pure glyphosate at the same concentration. This suggests that this reaction could be caused by additives in the formulation, which warrants further investigation. We however found no effect of Glyfonova® on levels of serum IL-6, which is known to induce the acute phase protein.

In conclusion, we have shown that pure glyphosate and the tested commercial formulation Glyfonova® had very limited effects on the gut microbial community composition in rats during a 2-week oral exposure trial at a concentration of 50xADI for humans. This is likely to be explained by sufficient bioavailability of aromatic amino acids in the gut environment, alleviating the effect of glyphosate blocking the Shikimate pathway. However, in cases of human malnutrition or in subjects consuming special (e.g. low protein) diets that may cause lower levels of available amino acids in the gut, a detrimental effect of glyphosate cannot be excluded.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.envpol.2017.10.016>.

Competing interests

The authors declare that they have no competing interests.

Author contributions

MIB, TRL and NBH conceived and designed the study. LNN, HMR, MEC, HLF and KB performed the experimental work. LNN, HMR, MEC, HLF, KB and MIB performed the analytical work. The manuscript was drafted by LNN and all authors contributed to interpretation of the results and approved the final manuscript.

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